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Mucosal vaccine delivery of antigens tightly bound to an adjuvant particle made from food-grade bacteria

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Abstract

Mucosal immunization with subunit vaccines requires new types of antigen delivery vehicles and adjuvants for optimal immune responses. We have developed a non-living and non-genetically modified gram-positive bacterial delivery particle (GEM) that has built-in adjuvant activity and a high loading capacity for externally added heterologous antigens that are fused to a high affinity binding domain. This binding domain, the protein anchor (PA), is derived from the *Lactococcus lactis* AcmA cell-wall hydrolase, and contains three repeats of a LysM-type cell-wall binding motif. Antigens are produced as antigen–PA fusions by recombinant expression systems that secrete the hybrid proteins into the culture growth medium. GEM particles are then used as affinity beads to isolate the antigen–PA fusions from the complex growth media in a one step procedure after removal of the recombinant producer cells. This procedure is also highly suitable for making multivalent vaccines. The resulting vaccines are stable at room temperature, lack recombinant DNA, and mimic pathogens by their bacterial size, surface display of antigens and adjuvant activity of the bacterial components in the GEM particles. The GEM-based vaccines do not require additional adjuvant for eliciting high levels of specific antibodies in mucosal and systemic compartments.
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Keywords: Mucosal vaccine; Gram-positive enhancer matrix; *Lactococcus lactis*; Protein anchor; LysM; Bacterial particle; Surface display

1. Introduction

1.1. Mucosal immunization

Conventional vaccine delivery technologies are based on injection into the body of a mixture of protective components and an immunostimulatory agent. While this parenteral route of immunization has proven to be effective, it often lacks stimulation of local immunity at the site, where most pathogens enter the body: the mucosal surface. The most effective way to induce mucosal immunity (secretory IgA) is to administer a vaccine directly to the mucosal surface. M-cells in the upper nasopharyngeal tissue are capable of antigen uptake and subsequent transport to the professional antigen presenting cells (APCs) which can elicit local and distal mucosal as well as systemic immune responses [1]. Peyer's patches in the intestinal tract (GALT) and nasopharyngeal-associated lymphoreticular tissue (NALT) in the nasal cavity are part of the common mucosal immune system, capable of effectively inducing antigen-specific T-helper (Th), cytotoxic T lymphocytes (CTLs), and IgA B-cell responses [2,3]. In addition to stimulating local immunity, mucosal vaccination has the benefits of needle-free administration, reduced side-effects, easy boosting, and can be used for mass vaccination. Furthermore, it is increasingly

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more difficult to implement new injectable vaccines into existing pediatric vaccination programs for which the number of injections is limited; a problem that can be circumvented by mucosal vaccination.

In spite of the ease of mucosal administration, safe, effective, and affordable carrier and adjuvant systems that are essential for these types of vaccines are still lacking. Bacterial carrier systems are easy to produce and have built-in adjuvant activity. Attenuated strains of bacterial pathogens such as *Salmonella typhi*, *Mycobacterium bovis*, and *Bordetella pertussis* are being developed as recombinant vectors for mucosal immunization, but suffer from the disadvantage that they tend to disseminate in the body [1], have the risk of transferring recombinant DNA to other organisms and can be hazardous in immunocompromised people. Bacterial ghosts made from gram-negative bacteria like *Escherichia coli* reduce some of these risks. They are non-living and can be used as antigen delivery particles [4,5]. These ghosts are empty cell envelopes made by protein-E-mediated lysis which have retained almost all morphological and structural features of the natural cell. They contain some well-known immune stimulating compounds such as lipopolysaccharide (LPS), Lipid A, and peptidoglycan and are capable of stimulation of both cellular and humoral arms of the immune system [6]. However, the presence of LPS in the gram-negative cell wall has been suggested as a potential problem for human applications, although it did not induce fever in test animals [7]. In any case, these bacterial ghosts are derived from genetically modified organisms (GMO) and the presence of residual amounts of recombinant DNA in non-lysed cells may constitute an obstacle for the application of this carrier system in mucosal vaccines.

The antigen display and delivery system described here, named the MimoPath system, is based on non-genetically modified gram-positive bacteria. It consists of non-living bacterial shaped delivery particles with adjuvant properties, which are loaded with antigens containing a cell-wall binding domain, called the protein anchor (PA, Fig. 1) [8]. The particles, referred to as gram-positive enhancer matrix (GEM), are made from acid-pretreated *Lactococcus lactis* bacteria. These GEM particles retain their original size and structure, of about 1 μm, and are thus ideally sized for uptake by the M-cells on the mucosal surface. Antigens can be produced as fusion proteins with the PA. These antigen–PA fusions bind non-covalently with high affinity to the peptidoglycan from GEM particles when mixed in solution (in trans binding). The result is a vaccine that mimics pathogens, producing a bacterial particle containing peptidoglycan with antigenic proteins exposed on its surface. An important advantage of the MimoPath system compared with recombinant bacterial delivery systems is the lack of recombinant DNA. Thus, it minimizes the risks associated with the spread of recombinant DNA into the environment, which is especially relevant in the case of wide-spread mucosal administration of vaccines.

1.2. The protein anchor

The protein anchor (PA) is made up of three LysM motifs of about 45 amino acids, separated by spacer regions. The LysM motif is a common module found in many cell-wall degrading enzymes and proteins involved in bacterial pathogenesis and is often present in multiple repeats [9–11]. It has been proposed that the LysM-type cell-wall binding domain binds non-covalently to peptidoglycan of various gram-positive bacteria [12]. The LysM motif has also been found in a number of eukaryotic proteins [10], and it was recently demonstrated that LysM domains are involved in recognition of symbiotic bacteria by the roots of plants [13–15]. In *L. lactis*, following secretion of the AcmA cell-wall hydrolase, the PA directs the protein to the cell wall. Hybrid PA fusion proteins, like MSA2, β-lactamase, α-amylase, viral capsid proteins and FedF-PA fusions show similar properties [8,16–18].

1.3. The GEM particle

In all experiments with PA fusion proteins, it was observed that only a small amount of the secreted PA fusions bind to the *L. lactis* producer cells. Most of the

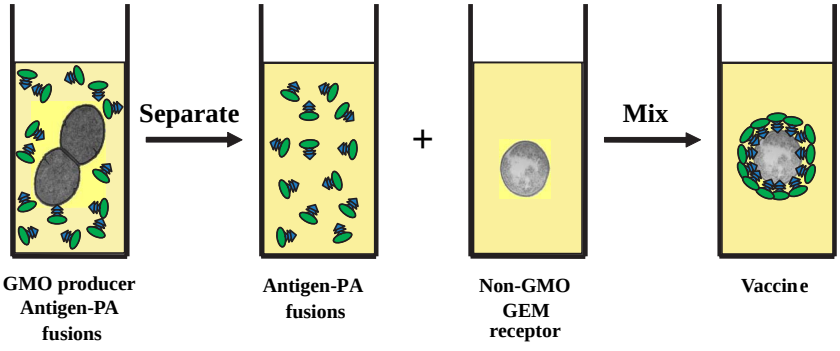

Fig. 1. Production of a vaccine: binding of PA fusions to GEM particles. In a two-step procedure, antigens containing a protein anchor domain (PA) and a gram-positive enhancer matrix (GEM) particle of bacterial origin are used to make a vaccine. First, the antigen–PA fusions are produced in the medium and separated from the producer cells. Next, GEM particles and cell-free medium are mixed. The PA directs the antigens to the surface of the GEM particles, where they bind tightly to the peptidoglycan layer.

secreted PA-fusion protein remains in the culture supernatant. In a number of cases, it was demonstrated that the PA fusions in the growth medium can be used to bind to non-producer *L. lactis* cells, albeit again in small amounts [12,18]. Steen et al. [12] discovered that boiling of *L. lactis* whole cells in trichloroacetic acid (TCA) followed by washing and neutralization prior to binding, dramatically increased the amount of MSA2-PA that can be bound to the cells. Other acid-pretreated gram-positive bacteria like *Lactobacillus* ssp. also showed increased binding capacity, suggesting that this method is applicable for production of GEM particles from a wider range of bacteria [12]. Since *L. lactis* is a GRAS organism (generally recognized as safe), lacks endotoxic LPS and lactococcal GEM particles do not contain recombinant DNA, safety issues are anticipated to be limited.

Binding of the PA to GEM particles is non-covalent, but very strong and approximately one million PA molecules can be bound on to one particle [31]. So far, only treatment with SDS sample buffer or 8 M LiCl was able to partially release the PA (patent WO02101026 [19]).

1.4. The MimoPath display and delivery system

Efficient and selective cell-surface binding to GEM particles occurs after addition of cell-free culture medium from recombinant producer strains that secrete chimeric PA-fusion proteins, without the need for additional purification steps. Bosma et al. [31] have shown the versatility and flexibility of this system by functional display of two enzymes, α-amylase and β-lactamase, in different ratios onto the surface of GEM particles. PA-mediated surface display differs in a number of aspects from bacterial surface display and delivery systems known to date [20]. First, PA-containing fusions are added from the outside to gram-positive cells (in *trans* binding). Second, the strain for production of hybrid PA-containing proteins and the host strain for surface display are different, allowing the use of a non-GMO delivery vehicle. In contrast, current bacterial display systems rely on recombinant host strains, in which the anchoring domain is covalently attached to the cell surface during translocation [21]. Third, multiple PA fusions can be bound on to one particle in adjustable ratios, allowing the production and delivery of multivalent vaccines. Alternatively, monovalent vaccines can be mixed together in different ratios facilitating flexible vaccine compositions. Finally, PA fusions can be bound to living as well as to non-living gram-positive bacteria.

Here, we describe the production and use of GEM-based vaccines. The construction and expression of antigen–PA fusions and the construction of GEM particles from *L. lactis* are described in detail. Furthermore, we show that GEM-bound PA is highly stable for long periods of time. Also, the use of a GEM-based vaccine for induction of serum IgG and local secretory IgA is demonstrated in an animal model.

2. Detailed methods

2.1. General molecular biology

Strains and plasmids used in this study are listed in Table 1. *L. lactis* strains were grown in standing cultures at 30 °C in M17 broth (Oxoid, The Netherlands) containing 0.5% glucose (w/v) (GM17) and, when necessary, supplemented with 5 μg/ml chloramphenicol ($GM17Cm_5$). Phosphate-buffered saline (PBS) contained 58 mM Na_2HPO_4, 17 mM NaH_2PO_4, and 68 mM NaCl at pH 7.2. Enzymes and buffers were purchased from New England Biolabs (USA) or Roche (The Netherlands). Electro-transformation of *L. lactis* was carried out as described previously [22] using a Bio-Rad Gene Pulser (Bio-Rad, The Netherlands).

2.2. Antigen–protein anchor fusions

For easy production of antigen–PA fusions, the pPA90 vector was constructed. This vector contains the following features (Fig. 2): the lactococcal pNZ212 origin of replication; a chloramphenicol acetyltransferase gene for selection; the P_{nisA} promoter to direct inducible expression of PA fusions [23]; the lactococcal USP45 secretion signal to drive secretion [24]; the gene fragment encoding the PA (the C-terminal 218 amino acid cell-wall binding domain of AcmA of *L. lactis* [25] including its stop codon); and a transcriptional

Table 1
Bacterial strains and plasmids

Strains and plasmids	Relevant phenotype(s) or genotype(s)	Source or reference
L. lactis MG1363	Plasmid-free derivative of NCDO712	[28]
L. lactis NZ9700	Nisin producer	[29]
L. lactis NZ9000	MG1363 *pepN*::*nisRK*, allows nisin inducible expression	[30]
L. lactis NZ9000(Δ*acmA*)	Derivative of NZ9000 lacking *acmA*	[8]
L. lactis PA1001	Derivative of NZ9000 lacking *acmA*and *htrA*	[31]
pPA3	Cm^r, pNZ8048 derivative containing *c-myc*, the *acmA* PA (nt 835–1492) under control P_{nisA} and $usp45_{ss}$ (PA3)	[12]
pPA90	Cm^r, pPA3 derivative lacking the *c-myc* epitope (PA90)	This study

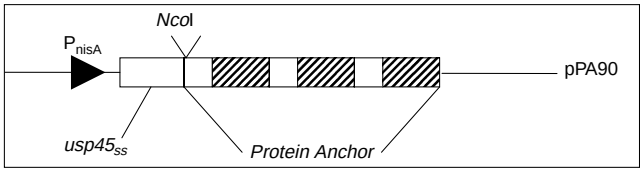

Fig. 2. The pPA90 vector. Schematic representation of a part of the general display vector pPA90. *Abbreviations:* P_{nisA}, inducible *nisA* promoter; $usp45_{ss}$, *usp45* signal sequence; *Nco*I, restriction site for integration of antigen genes; hatched blocks depict the LysM repeat sequences.

terminator is present 445 bp downstream of the PA gene fragment. Genes can be inserted in the unique *Nco*I site, resulting in a transcriptional fusion with the Met residue located at position +5 of the mature secreted protein.

2.3. Overexpression of PA-fusion proteins in L. lactis

Although it is possible to produce PA-fusion proteins secreted by *Bacillus subtilis* or by intracellular expression in *L. lactis* (data not shown), secretion by *L. lactis* into the medium is the standard production method. PA-fusion proteins can be produced by *L. lactis* PA1001, a MG1363 derived strain lacking *acmA* and *htrA* genes, and containing the *nisRK* genes needed for nisin induced expression. The housekeeping protease HtrA has been shown to degrade the AcmA cell-wall binding domain [25–27] and strains lacking *htrA* are more efficient in production of PA containing proteins. Lack of the *acmA* gene causes the bacteria to grow in strings, since the hydrolase needed for cell separation is absent [25].

In a typical production experiment, *L. lactis* PA1001 containing a pPA90-derived vector coding for an antigen–PA fusion protein, was grown overnight in $GM17Cm_5$ at 30 °C in standing cultures. Subsequently, the cells were diluted 100-fold in 1 l of fresh pre-warmed (30 °C) $GM17Cm_5$. At a cell density OD_{600} of 0.5, expression of the PA-fusion protein was induced by addition of 1 ml of cell-free nisin-containing supernatant produced from an overnight culture of *L. lactis* NZ9700. The induction was repeated at OD600 of 1.0. After each addition of nisin-containing supernatant the culture was thoroughly mixed and then left standing to grow at 30 °C for a total of 24 h before cells and supernatant were separated by centrifugation for 10 min at 13,000*g*. The supernatant was either used directly for binding after filter-sterilization (0.45 μm), or concentrated first with a VivaFlow 200 (10 kDa molecular mass cut off) and filter-sterilized again. Concentration of the culture supernatant may be used, when production levels are low and in general this increases the amount of bound PA-fusion protein to GEM particles.

2.4. Production of the GEM particles

Chemical pre-treatment of *L. lactis* MG1363 to generate GEM particles was performed as follows. Cells from a fresh overnight culture were diluted 100-fold in 500 ml of fresh GM17 and the standing culture was left to grow for 16 h at 30 °C. Cells were collected by centrifugation (10 min, 13,000*g*) and washed once by the addition of 250 ml sterile distilled water, vortexed, and centrifuged. Subsequently, 100 ml acid solution was added to the pellet and the cell suspension was placed in boiling water for 30 min in a tube under atmospheric pressure. When larger volumes were used, 1 l bottles containing a maximum of 300 ml cell suspension were steamed in a sterilizing pressure cooker with an unscrewed lid for 30 min at atmospheric pressure. The acid solution contained either 0.1 M HCl, 0.6 M TCA or 0.05 M H_2SO_4 in water which corresponds with an acidity of pH 1. The pH is known to be the critical factor in successful GEM production, not the type of acid. Next, the cells were washed three times in 250 ml sterile PBS with vigorous vortexing. After the last washing step, cells were taken up in 50 ml PBS. The number of GEM particles per milliliter was determined microscopically with a Bürker–Turk counting chamber. One unit (U) is defined as 2.5×10^9 GEM particles and standard stock solutions containing 10 U/ml were stored at −80 °C. The dry weight of GEM particles is approximately 250 μg/U. The GEM particles generated in this way are capable of binding 10–15 times more PA proteins than untreated *L. lactis* cells. Furthermore, the morphology of these particles remains almost identical to their untreated precursor cells. The DNA content (non-recombinant) is reduced by a factor of one million, as determined by real-time quantitative PCR, and the treatment resulted in complete killing of all cells (T. Bosma, submitted for publication).

2.5. Binding of PA-containing proteins to GEM particles

Protein anchor fusion proteins quickly bind to GEM particles when added together in solution. The standard procedure is to add one unit GEM particles to 2 ml PA-containing culture supernatant and slowly mix it on a rotating blood suspension mixer for up to 30 min. When concentrated, purified PA is used, binding occurs in seconds during mixing. After binding, GEM particles are washed in PBS and resuspended in their original volume. We were unable to demonstrate, by quantitative rtPCR, the presence of recombinant DNA in GEM-based vaccines that could have been included by non-specific binding of recombinant plasmid DNA, released by lysis of producer cells.

2.6. Stability of bound protein

Stability of the GEM-bound protein anchor over time was determined under the influence of different storage conditions like temperature, buffer components, and drying. *L. lactis* PA1001 (pPA3) was induced with nisin as described before to produce the PA3 protein. PA3 is similar to PA90 but contains an N-terminal *c-myc* epitope for easy immunological detection. The GEM particles were made from *L. lactis* NZ9000Δacma using TCA as acid. For each group, 2 ml of PA3-containing supernatant was bound to one unit GEM particles. The GEM particles were washed with water or PBS and stored at three different temperatures (−80, 4 °C, and room temperature [10–30 °C]) in solution or as freeze-dried powder. After 1, 2, 3, 6, and 12 months the particles were subjected to SDS–PAGE and Western blotting to determine the amount and stability of bound PA3 (Fig. 3). After one year, PA3 was still bound to GEM particles and remained intact.

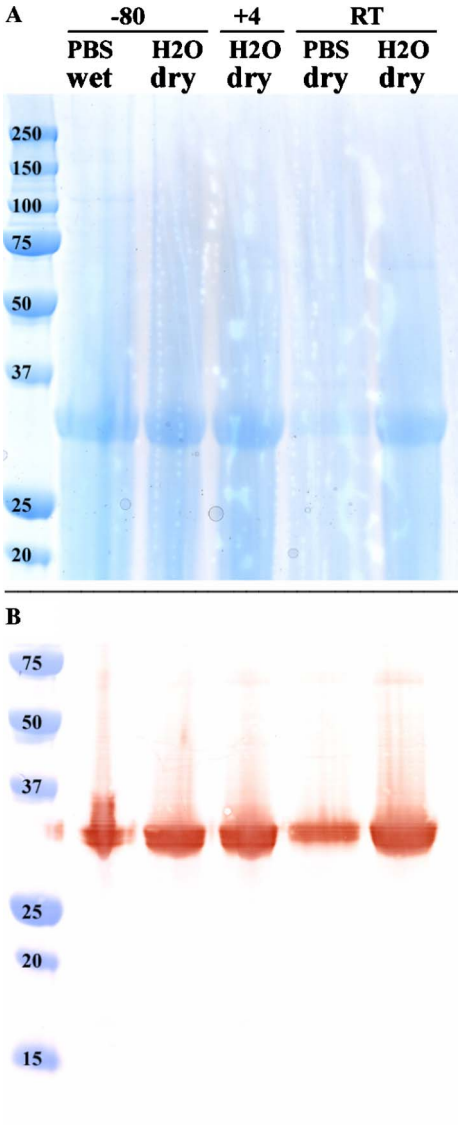

Fig. 3. Stability of GEM-PA after one year of storage. (A) The PA3 protein of approximately 26 kDa is visible as a band of slightly shorter running distance on this Coomassie brilliant blue-stained protein gel with samples from GEM-PA3. Samples were kept for one year at different storage conditions, indicated above the figure. The intensity of all PA3 signals is approximately equal, except for the PA3 in the lane with GEM-PA3 that was freeze dried in PBS and stored at room temperature, which could not be fully resuspended. The background smear that is present in all lanes originates from residual degraded proteins in the GEM particles. (B) Antibodies against the *c-myc* epitope present in this PA3 were used to detect the PA in a Western blot containing the same samples as in (A).

The best freeze-dried formulation was obtained when the GEM particles were dried in water. Freeze drying in PBS resulted in a hard pellet that was difficult to resuspend. When the particles were examined by microscopy, no visible difference in particle size was observed (data not shown). These results show that GEM-bound PA can be stored as a freeze-dried powder at room temperature for at least one year without any sign of degradation of its components. This indicates that a cold-chain route for delivery of GEM-based vaccines can be avoided.

3. Applications

3.1. Induction of local and systemic antibodies by mucosal immunization using the MimoPath system

The use of GEM-based vaccines as efficient delivery system for mucosal vaccination was shown by induction of serum IgG and secretory IgA in nasally immunized mice by Audouy et al. (S. Audouy, submitted for publication). Two pneumococcal antigens, SlrA and PpmA, were produced as PA fusion proteins and separately bound to GEM particles, resulting in two monovalent vaccines that contained either 73 μg/U SlrA-PA or 17μg/U PpmA-PA antigen. Two groups of mice, each receiving one monovalent vaccine, were immunized nasally with 3 doses given at 10-day intervals. Each dose contained one unit (2.5×10^9) GEM particles. The mice were killed 20 days after the last immunization. At killing blood samples, nose and lung lavages (with 0.5 and 1 ml PBS containing protease inhibitor, respectively) were collected and the amount of antigen-specific IgG and IgA was determined using ELISA techniques. For this purpose, ELISA microtiter plates were coated with purified His-tagged variants of the two antigens (0.2 μg/well). Plates were washed with PBS (pH 7.4) with 0.02% (v/v) Tween 20, then incubated 1 h with 1% (w/v) BSA in PBS/Tween. Sera were diluted, added to the plates in threefold dilutions and incubated for 2 h at room temperature. After washing, the alkaline phosphatase conjugated secondary antibody directed to mouse IgA or IgG was incubated for 1.5 h at a dilution of 1:5000. Colorimetric reaction was obtained by addition of *p*-nitrophenyl phosphate substrate. As shown in Table 2, both GEM-based vaccines were capable of stimulating high levels of antigen-specific secretory IgA as well as circulating serum IgG antibodies against both displayed antigens.

4. Discussion

We have described a method to make a GEM-bound antigen delivery vehicle, called the MimoPath system, using a number of steps. First, GEM particles are produced by hot acid pre-treatment of gram-positive *L. lactis* bacteria. The bacterial-sized GEM particles are devoid of most proteins, DNA and cellular components and show enhanced binding capacity for proteins containing the

Table 2
Secretory IgA and serum IgG levels in vaccinated mice (means ± SEM)

	Anti-SlrA ($n = 3$)	Anti-PpmA ($n = 4$)
Nose IgA (ng/ml)	256 (±108)	110 (± 38)
Lung IgA (ng/ml)	45 (±20)	28 (± 21)
Serum IgG (μg/ml)	178 (±32)	207 (± 102)

Two groups of mice, each receiving one monovalent vaccine, were immunized nasally with 3 doses given at 10-day intervals. Antigen-specific IgG and IgA levels were measured 20 days after the last immunization in blood samples, nose and lung lavages with ELISA (S. Audouy, submitted for publication). No specific antibody was detected in mice that received PBS only.

peptidoglycan-binding protein anchor. Second, antigen–PA fusions are secreted into the growth medium of an *L. lactis* producer strain. The cell-free medium can be used directly, or can be concentrated by ultrafiltration before binding. No inactivation procedures that denature relevant immunogenic determinants are employed. Finally, GEM particles and PA-fusion protein containing medium are mixed and as a result the PA fusions bind tightly to the peptidoglycan present in the GEM particles. Immobilization of PA fusions is fast and easy without the need for chemical treatments or extensive purification steps. Multivalent vaccines can be made by attachment of different antigen–PA fusions to the same particle, or by a mixture of monovalent vaccines. In our laboratory, we have successfully made and used a pentavalent vaccine against *Streptococcus pneumoniae* using both formulations (data not shown).

Vaccines based on the MimoPath delivery system circumvent some of the drawbacks associated with the use of genetically modified bacterial delivery systems. These GEM-based vaccines would also be very suitable for use in developing countries: they enable needle-free administration, allow easy immunization of large populations, and most likely they can be transported and stored for a long period without the need for a cold chain. Furthermore, these vaccines are easy to produce, can induce an immune response at distant mucosal sites (data not shown), and are based on a GRAS organism that is also present in dairy products for human consumption. Currently, we are using the MimoPath system to evaluate its efficacy in animal models of diseases from bacterial, viral, and parasitic origin.

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